

PTO 03-1457

CY=JP DATE=19910228 KIND=A
PN=03-047097

HYBRIDIZATION METHOD, GENE MUTATION DETECTION METHOD
USING IT AND APPARATUS THEREOF

[Haiburidaize-shon hoho, kore wo mochiita
idenshi hen'i kenshutsu hoho oyobi sono sochi]

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UNITED STATES PATENT AND TRADEMARK OFFICE
Washington, D.C. January 2003

Translated by: FLS, Inc.

PUBLICATION COUNTRY (10) : JP

DOCUMENT NUMBER (11) : 03047097

DOCUMENT KIND (12) : A

PUBLICATION DATE (43) : 19910228

PUBLICATION DATE (45) :

APPLICATION NUMBER (21) : 01178933

APPLICATION DATE (22) : 19890713

ADDITION TO (61) :

INTERNATIONAL CLASSIFICATION (51) :

DOMESTIC CLASSIFICATION (52) : C12Q 1/68; C12M 1/00; G01N
27/447; G01N 27/26

PRIORITY COUNTRY (33) :

PRIORITY NUMBER (31) :

PRIORITY DATE (32) :

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TITLE (54) : HYBRIDIZATION METHOD, GENE
MUTATION DETECTION METHOD USING
IT AND APPARATUS THEREOF

FOREIGN TITLE [54A] : HAIBURIDAIZE-SHON HOHO, KORE WO
MOCHIITA IDENSHI HEN'I KENSHUTSU
HOHO OYOBI SONO SOCHI

Specifications

/757*

1. Title of the Invention

Hybridization Method, Gene Mutation Detection Method Using It and Apparatus Thereof

2. Claim

1. A method for hybridizing a nucleic acid sample with a nucleic acid probe; said method for hybridizing a nucleic acid sample characterized by fixing a nucleic acid probe in an electrophoresis carrier and allowing a nucleic acid sample to migrate into the electrophoresis ["electric" in original] carrier by electrophoresis.

2. A method for detecting a gene mutation using a hybridization reaction on a nucleic acid sample with a nucleic acid probe; said method for detecting a gene mutation characterized by fixing the nucleic acid probe in an electrophoresis carrier, allowing the nucleic acid sample to migrate in the electrophoresis carrier by electrophoresis, performing a hybridization reaction, and allowing the above-mentioned nucleic acid sample bonded to the above-mentioned nucleic acid probe to migrate by electrophoresis to remove it from the above-mentioned electrophoresis carrier.

3. A method for detecting a gene mutation using a hybridization reaction on a nucleic acid sample with a nucleic acid probe; said method for detecting a gene mutation characterized by fixing the nucleic acid probe in an electrophoresis carrier, allowing the nucleic

¹Number in the margin indicates pagination in the foreign text.

acid sample to migrate into the electrophoresis carrier by electrophoresis, performing a hybridization reaction, then heating the aforesaid electrophoresis carrier, and subsequently allowing the above-mentioned nucleic acid sample bonded to the above-mentioned nucleic acid probe to migrate by electrophoresis to remove it from the above-mentioned electrophoresis carrier.

4. A method for detecting a gene mutation using a hybridization reaction on a nucleic acid sample with a nucleic acid probe; said method for detecting a gene mutation characterized by fixing the nucleic acid probe in an electrophoresis carrier, allowing the nucleic acid sample to migrate into the electrophoresis carrier by electrophoresis, performing a hybridization reaction, migrating the above-mentioned nucleic acid sample bonded to the above-mentioned nucleic acid probe by electrophoresis and removing it from the electrophoresis carrier, and further, migrating a labeled nucleic acid probe into the electrophoresis carrier by electrophoresis, performing a hybridization reaction, allowing the above-mentioned labeled nucleic acid probe bonded to

the above-mentioned nucleic acid probe to migrate by /758 electrophoresis to remove it from the electrophoresis carrier, and subsequently detecting the label of the labeled nucleic acid probe bonded to the above-mentioned nucleic acid sample.

5. A method for detecting a gene mutation using a hybridization reaction on a nucleic acid sample with a nucleic acid probe; said method for detecting a gene mutation characterized by fixing the

nucleic acid probe in the electrophoresis carrier, allowing the nucleic acid sample to migrate into the electrophoresis carrier by electrophoresis, performing a hybridization reaction, then heating the aforesaid electrophoresis carrier, subsequently allowing the above-mentioned nucleic acid sample bonded to the above-mentioned nucleic acid probe to migrate by electrophoresis to remove it from the above-mentioned electrophoresis carrier, and further, allowing a labeled nucleic acid probe to migrate into the electrophoresis carrier by electrophoresis, performing a hybridization reaction, then heating the above-mentioned electrophoresis carrier, subsequently allowing the above-mentioned labeled nucleic acid probe bonded to the above-mentioned nucleic acid probe to migrate by electrophoresis to remove it from the electrophoresis carrier, and subsequently detecting the label of the labeled nucleic acid probe bonded to the above-mentioned nucleic acid sample.

6. The method for detecting a gene mutation of claim 4 or 5 characterized by the label being a phosphor or a dye, and detecting these labels in the electrophoresis carrier.
7. The method for detecting a gene mutation of claim 4 or 5 characterized by the label being an enzyme, and detecting the phosphor or dye produced by an enzymatic reaction using the concerned enzyme in the electrophoresis carrier, or allowing it to migrate outside the above-mentioned electrophoresis carrier to detect it.
8. An apparatus for detecting a gene mutation using a hybridization

reaction on a nucleic acid sample with a nucleic acid probe; said apparatus for detecting a gene mutation characterized by being provided with an electrophoresis carrier in which the nucleic acid probe is fixed in order to hybridize the nucleic acid sample, a direct current voltage application means for applying a direct current voltage to the electrophoresis carrier to which the above-mentioned nucleic acid probe is fixed through an electrolytic solution on the positive electrode side and an electrolytic solution on the negative electrode side, and a measurement means for measuring the absorption of fluorescence or light in the above-mentioned electrophoresis carrier or above-mentioned electrolytic solution on the positive electrode side.

9. The apparatus for detecting a gene mutation of claim 8 characterized by the measurement means being a means for measuring the absorption of fluorescence or light in the electrolytic solution on the positive electrode side; and providing a membrane through which the electrolytic solutions pass but not the phosphor or the dye in order to concentrate the phosphor or dye in the electrolytic solution on the positive electrode side measured by the aforesaid measurement means.

10. The apparatus for detecting a gene mutation of claim 8 or 9 characterized by being provided with a means for controlling the temperature of the above-mentioned electrophoresis carrier.

11. An electrophoresis carrier on which a nucleic acid probe, which is used in a hybridization method on a nucleic acid sample with a

nucleic acid probe or in a method for detecting a gene mutation using a hybridization reaction on a nucleic acid sample with a nucleic acid probe, is fixed.

3. Detailed Specifications

(Field of Industrial Utilization)

The present invention relates to a method for hybridizing a nucleic acid sample, a method for detecting a gene mutation using this method and an apparatus thereof, and in particular, a high-speed and easily automated method for detecting a gene mutation.

(Prior Art)

A conventional method for detecting a gene mutation using a hybridization reaction in which either one of a nucleic acid (DNA or RNA) sample or DNA (or RNA) probe (a DNA (or RNA) fragment having a base sequence complementary with the target DNA (or RNA)) is fixed to a solid phase is disclosed in Proc. Natl. Acad. Sci. USA, 80, (1983), pp. 278-282.

In this method, a hybridization reaction is performed by transcribing and fixing a DNA fragment samples separated by molecular weight by electrophoresis onto a nitrocellulose membrane and subsequently immersing this membrane in a solution containing a DNA probe. Due to the hybridization reaction, the higher the complementation of the base sequence, the stronger the DNA fragment sample is bonded to the DNA probe and it does not dissociate even at high temperatures. Therefore, washing is performed at a temperature so that when the DNA fragment sample

has perfect complementation with the DNA probe, it does not dissociate or it dissociates when the complementation is incomplete. When the DNA fragment sample has perfect complementation with the DNA probe, the DNA probe remains bonded to the film as is and is detected, but if it does not, the DNA probe is not detected because it is washed away from the membrane. As above, in this method, whether or not the DNA fragment sample has perfect complementation with the DNA probe can be judged. Therefore, by making the DNA probe from a DNA fragment having perfect complementation with a normal target gene, whether the target gene in the DNA fragment sample is a normal one or an abnormal one containing a mutation, such as point mutation, insertion or deletion, can be detected.

(Problems Which the Invention Intends to Solve)

There was a problem with the above-mentioned conventional method because the reaction rate was slow since the hybridization reaction with the DNA fragment sample fixed to the nitrocellulose membrane (solid phase) occurred by passive dispersion of the DNA probe in solution. There was an additional problem because actions which were difficult for automation of injection and elimination of the solution was contained at the time of the reaction or washing.

The object of the present invention is to obtain a high-speed, easily automated hybridization method wherein the hybridization reaction rate is fast, there are no actions which are difficult for automation of the injection, elimination, and the

like of a solution, and a method for detecting a gene mutation using this method and an apparatus used therein.

(Means Used to Solve the Problems)

In order to achieve the above-mentioned object, in the present invention, the hybridization reaction and washing are performed by fixing the DNA probe in the electrophoresis carrier, arranging two electrodes above and below that carrier with the aid of a buffer, and the nucleic acid fragment sample and the like are forced to migrate by electrophoresis.

Namely, the present invention is a method for hybridizing a nucleic acid sample with a nucleic acid probe; this method for hybridizing a nucleic acid sample characterized by fixing a nucleic acid probe in an electrophoresis carrier and allowing a nucleic acid sample to migrate into the electrophoresis [electric in original] carrier by electrophoresis. In this hybridization method, the nucleic acid sample was forced to migrate over the electrophoresis carrier in which the DNA probe is fixed; hence, the hybridization reaction is faster than in the above-mentioned conventional method, and this reaction can be completed in a short time.

Furthermore, the present invention is a method for detecting a gene mutation using a hybridization reaction on a nucleic acid sample with a nucleic acid probe; this method for detecting a gene mutation characterized by fixing the nucleic acid probe in an electrophoresis carrier, allowing the nucleic acid sample to

migrate in the electrophoresis carrier by electrophoresis, performing a hybridization reaction, and allowing the above-mentioned nucleic acid sample bonded to the above-mentioned nucleic acid probe to migrate by electrophoresis to remove it from the above-mentioned electrophoresis carrier.

The above-mentioned method for detecting a gene mutation can be performed by using two kinds of nucleic acid probes, i.e., a nucleic acid probe fixed to an electrophoresis carrier (an immobilized probe) and a 2nd labeled nucleic acid probe (a labeled probe) further hybridized with a nucleic acid sample bonded to the aforesaid immobilized probe. That is, this method for detecting a gene mutation can be performed by fixing the nucleic acid probe in an electrophoresis carrier, allowing the nucleic acid sample to migrate into the electrophoresis carrier by electrophoresis, performing a hybridization reaction, migrating the above-mentioned nucleic acid sample bonded to the above-mentioned nucleic acid probe by electrophoresis and removing it from the electrophoresis carrier, and further, migrating a labeled nucleic acid probe into the electrophoresis carrier by electrophoresis, performing a hybridization reaction, allowing the above-mentioned labeled nucleic acid probe bonded to the above-mentioned nucleic acid probe to migrate by electrophoresis to remove it from the electrophoresis carrier, and subsequently detecting the label of the labeled nucleic acid probe bonded to the above-mentioned nucleic acid sample.

Moreover, after performing the hybridization reaction, a process for heating the electrophoresis carrier can be added to each above-mentioned method. The heating temperature is preferably a temperature so as not to dissociate the nucleic acid sample when it has perfect complementation with the nucleic acid probe and dissociate it when there is no complementation or complementation is imperfect. This temperature varies widely depending on the lengths of the nucleic acid sample and the nucleic acid probe, the base sequence, and the mutation in the gene to be detected, but 55°C is preferable for example when a point mutation in a β-globin gene is detected with a nucleic acid probe having a 19-base length. Then, by heating this electrophoresis carrier, the accuracy of the method for detecting a gene mutation using a hybridization reaction between the nucleic acid probe and the nucleic acid sample can be increased.

Any detectable labeling substance can be used for the substance for labeling the above-mentioned labeled nucleic acid probe. It can be a radioisotope, such as ^{32}P , but preferably, a phosphor, dye, or an enzyme which forms a phosphor or dye in the reaction can be used. Fluorescein isothiocyanate (FITC), esterase, and the like are used as specific examples. Then, measurement of this phosphor or dye can be performed for either allowing the phosphor or dye to migrate into the above-mentioned electrophoresis carrier or outside the above-mentioned electrophoresis carrier by electrophoresis.

Furthermore, the present invention pertains to an apparatus for detecting a gene mutation for carrying out the above-mentioned method for detecting a gene mutation; it is an apparatus for detecting a gene mutation using a hybridization reaction on a nucleic acid sample with a nucleic acid probe; this apparatus for detecting a gene mutation characterized by being provided with an electrophoresis carrier in which the nucleic acid probe is fixed in order to hybridize the nucleic acid sample, a direct current voltage application means for applying a direct current voltage to the electrophoresis carrier to which the above-mentioned nucleic acid probe is fixed through an electrolytic solution on the positive electrode side and an electrolytic solution on the negative electrode side, and a measurement means for measuring the absorption of fluorescence or light in the above-mentioned electrophoresis carrier or above-mentioned electrolytic solution on the positive electrode side. Moreover, this apparatus for detecting a gene mutation can be provided with a membrane through which the electrolytic solutions pass but not the phosphor or the dye in order to concentrate the phosphor or dye in the electrolytic solution on the positive electrode side measured by the aforesaid measurement means when the measurement means is a means for measuring the absorption of fluorescence or light in the electrolytic solution on the positive electrode side. This membrane can be any membrane provided with the above-mentioned functions, but a porous glass membrane made of quartz can be used,

for example.

In addition, this apparatus for detecting a gene mutation can be provided with a control means for controlling the temperature of the above-mentioned electrophoresis carrier.

Furthermore, the present invention pertains to an electrophoresis carrier on which the above-mentioned nucleic acid probe, which is used in a hybridization method on a nucleic acid sample with a nucleic acid probe or in a method for detecting a gene mutation using a hybridization reaction on a nucleic acid sample with a nucleic acid probe, is fixed.

(Operation)

A DNA fragment sample is forced to migrate into the carrier by applying a direct current across two electrodes after adding the DNA fragment sample to the electrophoresis carrier. Thus, the hybridization reaction can be made faster than when the DNA fragment sample is dispersed passively.

Moreover, a DNA fragment sample not bonded or bonded weakly due to the hybridization reaction is removed by electrophoresis. Thus, a method suitable for automation can be realized without needed a washing operation by injection and elimination of a solution, and the like.

Furthermore, measurement of the fluorescence or light from the substance for labeling the hybridization reactant can be performed in either the above-mentioned electrophoresis carrier or the electrolytic solution on the positive electrode side. In addition,

when measurement is done in the latter electrolytic solution on the positive electrode side, the sensitivity of the measurement is enhanced by providing a membrane which concentrates the phosphor or dye.

(Practical Examples)

The present invention will now be explained more specifically through the practical examples. However, the present invention is not limited by these practical examples.

Practical Example 1

This practical example is explained through Figures 1(a) and (b).

First of all, an electrophoresis carrier 1 in which a DNA probe is fixed is prepared as follows. A DNA fragment (3'-GAGGACTCCTCTTCAGACG-5') perfectly complementary to the sequence of bases 14 to 32 from the 5' terminal of a human β -globin gene was synthesized for the DNA probe in a phosphoamide method that is currently used widely in the industry. However, in the final synthesis step, that is, the step in which the guanine (G) of the 5' terminal is added, an amino group was introduced at the 5' terminal of the DNA fragment in a method by L.M. Smith, et al. in which deoxyguanosine having an amino group at the 5' terminal was used instead of deoxyguanosine. Next, this DNA probe was prepared by high-performance liquid chromatography (HPLC), subsequently added to an aqueous 2.5% acrolein solution, and allowed to react

/761

for 30 minutes under cooling with ice. This was dialyzed well with a PBS buffer, after which a 5% acrylamide-N,N'-methylene bisacrylamide solution (acrylamide:N,N'-methylene bisacrylamide = 20:1), N,N,N',N'-tetramethyl ethylene diamine with a final concentration of 0.08%, and ammonium persulfate with a final concentration of 0.1% were further added, injected into a glass tube **2**, and gelled to get the electrophoresis carrier **1**.

The β -globin gene (β^A) of a healthy person and the β -globin gene (β^S) of a patient with sickle cell anemia wherein the 20th adenosine (A) from the 5' terminal mutated (point mutation) to thymine (T) and was cleaved with the restriction enzyme BamHI (a fragment of about 1,800 base pairs contained in the vicinity of the 5' terminal of the β -globin gene) were used for the DNA fragment samples.

The above-mentioned DNA fragment samples were subjected to heat denaturation to make a single strand DNA, which were then injected into the upper end of the electrophoresis carrier **1** in which a DNA probe maintained at 45°C by a temperature controller **3** was fixed, and a voltage from a direct current power source **10** was applied across the negative electrode **6** in an upper electrolytic solution bath **4** and the positive electrode **9** in a lower electrolytic solution bath **7**. Thus, the DNA fragment samples are forced by electrophoresis into the electrophoresis carrier **1**; hence, the hybridization reaction can proceed faster than when the

samples are dispersed passively without performing electrophoresis.

Next, the temperature of the electrophoresis carrier 1 was changed to 55°C by using the temperature controller 3, a voltage was subsequently applied across the two electrodes 6 and 9 again, and the DNA fragment sample dissociated because a perfect complementation with the DNA probe was not maintained was removed by electrophoresis.

Furthermore, a second DNA probe labeled with esterase was injected into the upper end of the electrophoresis carrier 1 after bringing the temperature of the electrophoresis carrier back to 45°C and this was subjected to electrophoresis. This DNA probe (labeled probe) was such that the 5' terminal of the DNA fragment (3'-CCACTTGCACCTACTTCAAC-5') synthesized in a phosphoamide method in the same way as the probe fixed to the electrophoresis carrier 1 (immobilized probe) was labeled with esterase, but it is complementary to a site different than the immobilized probe, that is, the sequence of bases 53 to 72 from the 5' terminal of the β -globin gene. Therefore, if the DNA fragment sample is bonded to the immobilized probe and remains in the electrophoresis carrier 1, the labeled probe is bonded to another site of the DNA fragment sample and also remains in the electrophoresis carrier 1, but if the DNA fragment sample does not remain, the labeled probe passes through without remaining in the electrophoresis carrier 1.

Lastly, FDA (fluorescein diacetate), which is a matrix for the

labeling enzyme esterase, was introduced into the upper end of the electrophoresis carrier **1** and subjected to electrophoresis in the same way, and the fluorescence of the fluorescent substance, fluorescein, produced by an enzymatic reaction was measured in the electrophoresis carrier **1**.

A 490 nm wavelength light obtained by passing the light emitted from a xenon lamp light source **11** through an interference filter **12**, subsequently condensed by a lens **13** was selected, and the electrophoresis carrier **1** was irradiated with this excited light. Light with a wavelength in the vicinity of 510 nm was selectively detected by a photomultiplier **20** after passing through a lens **17**, cut-off filter **18**, and interference filter **19** from a direction 90° to the excited light. Moreover, a window **16** was provided on the opposite side of a light incident window **14**, and the excited light that passed through the electrophoresis carrier **1** was guided to the outside; hence, influence by diffused light was minimized. The output from the photomultiplier **20** was amplified by an amplifier **21** and subsequently recorded by a recorder **22**.

As a result of measurement, when the DNA fragment sample was a β -globin gene (β^A) of a healthy person which did not contain a mutation and it had perfect complementation with the immobilized DNA probe, fluorescence was detected. However, when the DNA fragment sample was a fragment of β globin gene (β^S) of a patient with sickle cell anemia containing a mutation (point mutation) and

it had all but a complementation of only one base with the immobilized DNA probe, fluorescence was not detected. Upon performing the same measurement for confirmation by adding the immobilized DNA probe to a fragment with perfect complementation to the β^5 gene (3'-GAGGACACCTCTTGAGACG-5'), fluorescence was not detected when the DNA fragment sample was a fragment of the β^4 gene and fluorescence was detected when it was a fragment of the β^3 gene. In this way, the gene fragment containing a mutation and the gene fragment not containing a mutation could be discriminated as to whether or not fluorescence could be detected; hence, a mutation (point mutation) in the β -globin gene fragment could be detected.

/762

Moreover, an enzyme (esterase) was used as the labeling substance in this practical example to measure the fluorescence of the FDA produced by an enzymatic reaction, but a fluorescent substance, such as FITC, can be used as the labeling substance, and the fluorescence thereof can be measured directly without using an enzyme or enzymatic reaction.

According to this practical example as above, a high-speed and easily automated method for detecting a gene mutation and an apparatus could be realized.

Practical Example 2

The 2nd practical example will be explained next through Figure 2. The difference between this practical example and Practical Example 1 is that the fluorescence of the fluorescent substance,

fluorescein, is measured in the lower electrolytic solution **8**, not in the electrophoresis carrier **1**. In the last step of the aforesaid practical example, the FDA migrated into the electrophoresis carrier **1** by electrophoresis, and the fluorescent substance, fluorescein, produced by an enzymatic reaction by continuing electrophoresis further was subjected to electrophoresis in the lower electrolytic solution **8**. And so, the fluorescence of the fluorescein was measured in the lower electrolytic solution by using the apparatus shown in Figure 2.

According to this practical example, in addition to the same effects as the aforesaid practical example, the fluorescence of the fluorescein is measured not in the electrophoresis carrier in which there is a lot of diffused light and interfering fluorescence, but in an electrolytic solution in which there is hardly any of such light, there is an effect because a highly sensitive fluorescence measurement is possible.

Practical Example 3

The 3rd practical example will be explained next through Figure 3. The difference between this practical example and Practical Example 2 was that it was constituted with a small- capacity electrolytic solution bath **25** by arranging a porous glass membrane **24** mounted on a membrane holder **23** made of acrylic between the lower end of the electrophoresis carrier **1** and the lower electrolytic solution **8**. The above-mentioned porous glass membrane

24 was used to react tetramethoxysilane with methanol in an aqueous solvent in a sol-gel method. It is a quartz glass having properties where the electrolyte in the electrolytic solution permeates into it, but phosphor does not. Therefore, the phosphor, FDA, produced by an enzymatic reaction is concentrated in the small-capacity electrolyte bath **25**. In this practical example, the electrolytic solution containing the phosphor concentrated in the above-mentioned process passed through a guide hole **26** and was guided to a fluorescence cell **28** by using a pipette **27**. The pipette **27** was held by a vertical rotating mechanism **29**. The fluorescence of the phosphor in the fluorescence cell **28** was measured by the same optical system as shown in Figure 2.

According to this practical example, in addition to the same effect as in Practical Example 2, there is an effect where a highly sensitive fluorescence measurement is also possible because the fluorescent substance, FDA, is concentrated in a small volume of electrolytic solution.

(Advantages of the Invention)

According to the present invention, the DNA fragment sample is forced to migrate into the electrophoresis carrier by electrophoresis; hence, the hybridization reaction can be made faster than when it is passively diffused in a conventional method using nitrocellulose membrane, and it can be finished in a short time. Moreover, a DNA sample bonded or weakly bonded by a

hybridization reaction can be removed easily by electrophoresis without using a washing operation by injection, elimination, and the like of a solution. Therefore, according to the present invention, a high-speed and easily automated method for detecting a gene mutation can be realized. Furthermore, in the present invention, the measurement sensitivity can be enhanced by concentrating the phosphor or light of the labeling substance.

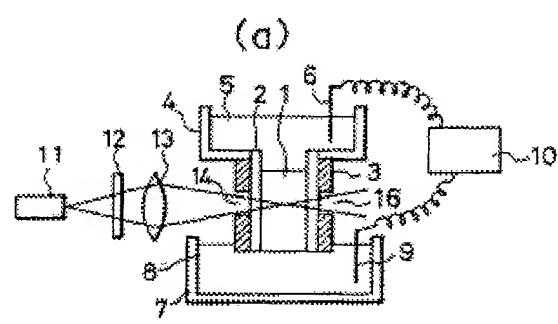
4. Brief Description of the Figures

Figures 1(a) and (b) are a longitudinal sectional view and cross sectional view of the apparatus used in the first practical example of the present invention, respectively; Figure 2 is a longitudinal sectional view of the apparatus used in the second practical example of the present invention; and Figure 3 is a partially enlarged longitudinal sectional view of the apparatus used in the third practical example of the present invention.

1: electrophoresis carrier; 2: glass tube; 3: temperature controller; 4: upper electrolytic solution bath; 5: upper (negative electrode side) electrolytic solution; 6: negative electrode; 7: lower (positive electrode side) electrolytic solution bath; 8: lower (positive electrode side) electrolytic solution; 9: positive electrode; 10: direct current power source; 11: light source; 12, 19: interference filters; 13, 17: lenses; 14: light incident window; 15: detection window; 16: window; 18: cut-off filter; 20: photomultiplier; 21: amplifier; 22: recorder; 23: membrane holder; 24: porous glass membrane; 25: small-capacity electrolytic solution

bath; 26: guide hole; 27: pipette; 28: fluorescence cell; 29:
vertical rotating mechanism

Figure 1



(b)

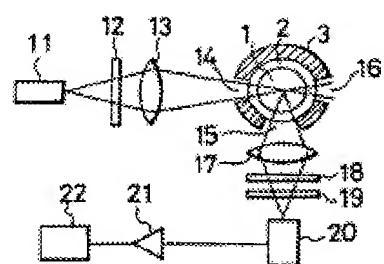


Figure 2

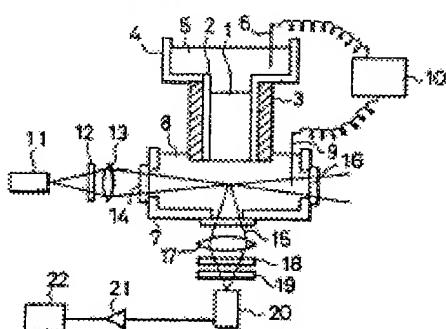


Figure 3

